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Title:

Combinatorial Targeting of TSLP, IL-25, and IL-33 in Type 2 Cytokine-driven Inflammation and Fibrosis

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One Sentence Summary: Although TSLP, IL-25, and IL-33 have emerged as important initiators of type 2 immunity, combined blockade of all three mediators may be needed to treat some forms of progressive type 2 cytokine driven inflammation and fibrosis.

Abstract: Thymic stromal lymphopoietin (TSLP), IL-25, and IL-33 are important initiators of type 2-associated mucosal inflammation and immunity. However, their role in the maintenance of progressive type 2 inflammation and fibrosis is much less clear. Here, using chronic models of helminth infection and allergic lung inflammation, we show that collective disruption of TSLP, IL-25, and IL-33 signaling suppresses chronic and progressive type 2 cytokine-driven inflammation and fibrosis. In a schistosome lung granuloma model or during chronic *S. mansoni* infection in the liver, individual ablation of TSLP, IL-25, or IL-33/ST2 had no impact on the development of IL-4/IL-13-dependent inflammation or fibrosis. However, significant reductions in granuloma-associated eosinophils, hepatic fibrosis, and IL-13-producing group 2 innate lymphoid cells (ILC2s) were observed when signaling of all three mediators was simultaneously disrupted. Combined blockade via mAb treatment also reduced IL-5 and IL-13 expression during primary and secondary granuloma formation in the lung. In a model of chronic house dust mite-induced allergic lung inflammation, combined mAb treatment did not decrease established inflammation or fibrosis. TSLP/IL-33 double-knockout mice treated with anti-IL-25 mAb during priming, however, displayed decreased inflammation, mucus production, and lung remodeling in the chronic phase. Together, these studies reveal partially redundant roles for TSLP, IL-25, and

IL-33 in the maintenance of type 2 pathology and suggest that in some settings, early combined targeting of these mediators is necessary to ameliorate progressive type 2-driven disease.

Main Text:

Introduction

Type 2 immunity is characterized by the production of the cytokines IL-4, IL-5, IL-9, and IL-13, which play diverse roles in the immune response (*1*). In addition to suppressing the pro-inflammatory activity of type 1 immune responses (*2*), type 2 immunity regulates wound healing (*3*), metabolic homeostasis (*4*), and immunity to several extracellular parasites (*5*). However, while the type 2 response exhibits many host protective functions, should these responses persist or become dysregulated, they can contribute to the development of disease. Chronic type 2 cytokine production underlies diseases including allergic asthma, atopic dermatitis, allergic rhinitis, ulcerative colitis, and many chronic fibroproliferative disorders (*6-9*). Therefore, a better understanding of the mechanisms that regulate the initiation, maintenance and resolution of type 2 immune responses could reveal novel approaches to treat a host of important human diseases.

Three predominantly epithelial cell-derived cytokines: thymic stromal lymphopoietin (TSLP), IL-25, and IL-33, have emerged as important initiators of type 2 immunity in mammals, and their expression during type 2 disease in humans is widely-documented (*10-15*). These alarmins are released from the epithelium and other local stromal compartments when cells are damaged or stressed by allergens, pollutants or pathogens and thereby trigger the production of

70 the canonical type 2 cytokines IL-5, IL-9, and IL-13 by human and mouse cells of the innate and
71 adaptive immune system (*16, 17*). TSLP targets dendritic cells (DCs), basophils, mast cells,
72 monocytes, natural killer T cells, and type 2 innate lymphoid cells (ILC2s) (*18-21*). In humans,
73 TSLP has been shown to induce naïve human $CD4^+$ Th2 cell responses, but only in the presence
74 of DCs (*22*). IL-25 and IL-33 exhibit similar Th2-inducing activity, but rather than targeting
75 DCs, myeloid cells, and Th2 cells, they largely promote type 2 immunity by stimulating ILC2s
76 as well as basophils, mast cells, and eosinophils. IL-33 will amplify antigen-dependent and -
77 independent effector responses from both human and mouse Th2 cells (*16, 17*). One recent
78 study revealed that IL-33 can enhance TSLP and DC-mediated human Th2 memory responses *in*
79 *vitro* suggesting the alarmins could play a role in maintaining immune responses (*23*). Although
80 TSLP, IL-25, and IL-33 have all been shown to promote type 2 immunity when overexpressed in
81 mice (*10-12*), the requirement for these cytokines in the development of type 2 immunity in
82 response to allergens and helminth parasites has been more variable, with some studies
83 identifying little to no role for TSLP, IL-25, or IL-33 when targeted individually (*24-28*). This
84 variability has been attributed to the redundant and overlapping functional activities of these
85 cytokines. IL-33 and IL-25 have both been shown to induce production of IL-13 by human ILCs
86 *in vitro*, for example (*29*). However, this theory has not been systematically investigated *in vivo*,
87 nor have the combined roles of the 3 cytokines been dissected in models of chronic type 2-
88 dependent disease.
89 In the present study, we utilized both genetic- and monoclonal antibody-based strategies
90 to investigate whether bi-functional or tri-functional targeting of TSLP, IL-25, and IL-33-
91 dependent signaling more effectively controls pathogenic Th2 responses than disrupting any of
92 the pathways individually. The roles of the 3 cytokines in the initiation and maintenance of

primary and secondary type 2 immune responses were investigated in both acute and chronic models of lung inflammation and during chronic helminth infection. These models involve innate-initiated pathways as well as the development of antigen-specific T cell responses that influence outcomes at later stages. A major goal was to investigate if type 2 cytokine-driven inflammation and fibrosis could be ameliorated more effectively if all three epithelial cytokines were targeted in combination. Moreover, in contrast to previous studies that have focused on their role in the "initiation" of type 2 immunity (*30*), our studies were also designed to investigate if TSLP, IL-25, and IL-33, either alone or in combination, are required for the "maintenance" of established type 2-driven disease, as this is the stage where most therapeutic strategies are initiated.

Results

Function of IL-25 during the initiation and maintenance of type 2 inflammation

We have previously shown that TSLP is not required for type 2-driven granuloma formation and fibrosis induced by the eggs of the helminth parasite *Schistosoma mansoni* (*26*). Another group has demonstrated that many helminths could bypass the need for TSLP in the development of type 2 responses by directly modulating dendritic cell function (*28*). However, the relative importance of IL-25 and IL-33 to the maintenance of established type 2-driven disease and the potential redundancy of these mediators has not been assessed. Therefore, we began by exploring the contribution of IL-25 in type 2-dependent inflammation and fibrosis by overexpressing IL-25 in mice that were injected i.v. with live *S. mansoni* eggs. Hydrodynamic delivery of an IL-25-expressing plasmid to naïve mice boosted IL-25 mRNA expression more

than 1000-fold in the liver (**Fig. 1A**). As observed in previous studies (*10*), corresponding increases in IL-4, IL-5, and IL-13 expression were observed in both the liver and the lung (**Fig. 1A**). When the IL-25-expressing plasmid was delivered 24 hours prior to exposure to *S. mansoni* eggs, the resulting granulomatous response to the eggs in the lung was exacerbated (**Fig. 1B**). Indeed, granuloma volume more than doubled in the IL-25 pre-treated mice, and their lesions contained many more eosinophils than control mice, which was likely due to type 2 cytokine induction in the lung (**Fig. 1C**). Goblet cell hyperplasia and mucus production were also augmented in lungs of mice treated with the IL-25 plasmid. The effects of IL-25 plasmid administration were reduced in IL-13Rα$^{-/-}$ mice, demonstrating that the IL-25-mediated increase in type 2-associated pathology was dependent on IL-4/IL-13-mediated signaling through the type II IL-4 receptor complex (**Fig. 1B**). Eosinophils accumulated following plasmid administration, however, likely explained by IL-25-driven IL-5 expression (**Fig. 1C**).

Although these studies established that IL-25 could exacerbate type 2 cytokine-driven pathology, they did not reveal whether endogenously expressed IL-25 was critical to the development of granulomatous inflammation and fibrosis. To clarify the role of IL-25 in both the initiation and maintenance of type 2-driven fibrosis, we used IL-25$^{-/-}$ mice in both primary (like Fig. 1B) and secondary i.v. *S. mansoni* egg challenge models (*31*). In these experiments, naïve or egg-sensitized IL-25$^{-/-}$ mice and wild-type littermates were challenged i.v. with live *S. mansoni* eggs and granuloma formation was quantified on day 7 post-challenge. Neither primary nor secondary granuloma formation was significantly reduced in the absence of IL-25 (**Fig. 1D**). The number of granulomatous eosinophils in each group was also indistinguishable during both primary and secondary challenges (**Fig. 1D, right panel**). Finally, to evaluate the requirement for IL-25 in a more chronic type 2 disease setting, we exposed wild-type and IL-25$^{-/-}$ mice to *S.*

mansoni cercariae and quantified granuloma volume, tissue eosinophilia, and fibrosis in the liver after 12 weeks of infection. Similar to the results in the lung (**Fig. 1D**), no significant change in type 2-dependent pathology was observed in livers of IL-25$^{-/-}$ mice compared with wild-type mice when chronically infected with *S. mansoni* (**Fig. 1E**).

Role of IL-33 in type 2 inflammation and fibrosis

Given that the inflammation and fibrosis induced by *S. mansoni* eggs in both the lung and liver were IL-4-, IL-13-, and IL-13Rα□-dependent but did not require IL-25 or TSLP (*26*), we next examined whether IL-33/ST2 receptor signaling was required in this setting. As observed in IL-25$^{-/-}$ mice, mice deficient in IL-33 showed no significant reduction in either primary (**Fig. 2A**) or secondary granuloma formation (**Fig. 2B**) when challenged i.v. with live *S. mansoni* eggs. In both models, type 2-driven fibrosis and eosinophilia were similar in wild-type and IL-33$^{-/-}$ mice. We also infected wild-type and IL-33$^{-/-}$ mice with *S. mansoni* cercariae and examined the development of type 2-dependent pathology in the liver at acute (week 9) and chronic (week 12) phases of infection. Although recent studies using hepatotoxic chemicals or schistosome egg-driven models have suggested that IL-33 expression is critical to the development of fibrosis in the liver (*32*), we observed no reduction in hepatic fibrosis in IL-33$^{-/-}$ mice at either time-point (**Fig. 2C**) and picrosirius red staining of liver sections (**Fig. 2D**). The number of eosinophils in the lesions and the diameter of granulomas were also similar in the absence of IL-33, confirming unimpaired type 2-driven inflammation (**Fig. 2E**). The marked type 2 cytokine response that normally develops in the livers of infected wild-type mice was also similarly observed in IL-33$^{-/-}$ mice, and in the case of IL-4 expression was even slightly increased (**Fig. 2F**), further suggesting

that IL-33 signaling is dispensable for the development of type 2 cytokine-driven pathology during both acute and chronic *S. mansoni* infection.

Disrupting TSLP, IL-25, and IL-33 signaling during *S. mansoni* infection

To investigate whether TSLP, IL-25, and IL-33 were playing redundant roles in the maintenance of type 2 cytokine-dependent granuloma formation and fibrosis, we developed strategies to disrupt all three cytokine pathways simultaneously. In initial studies, $TSLP^{-/-}$ mice were crossed with $IL\text{-}33^{-/-}$ mice to generate a double knockout mouse, and a highly effective neutralizing mAb was introduced to the double knockout mice to block IL-25. C57BL/6 mice were infected with *S. mansoni* cercariae, and the response in the absence of TSLP, IL-25, and IL-33 signaling was evaluated at acute (week 9) and chronic (week 12) phases of infection. We first measured TSLP, IL-25, and IL-33 gene expression and found each gene is constitutively expressed in whole liver tissue at detectable levels **(Fig. S1)**. These levels of expression do not change significantly during *S. mansoni* infection on a whole tissue level. In contrast to the studies in which individual cytokines were targeted, we observed a small yet significant decrease in granuloma volume in the triple deficient mice in the acute phase (**Fig. 3A**). This was also accompanied by a 25-30% decrease in hepatic fibrosis (**Fig. 3B**) and a small yet significant decrease in the number of granuloma-associated eosinophils (**Fig. 3C**). Interestingly, the decrease in pathology observed at week 9 was associated with a significant decrease in the frequency of IL-13-producing type 2 innate lymphoid cells (ILC2s) in the mesenteric lymph nodes (MLNs) (**Fig. 3D**), which is consistent with the ILC2-promoting activity of IL-25 and IL-33 (*33*). The frequency of ILC2s in the liver, however, was not significantly different between the two groups (**Fig. 3D, right panel**). Total leukocyte numbers were similar in the liver tissue and MLNs of both cohorts.

By 12 weeks post-infection, the decrease in IL-13-producing ILC2s in MLNs observed at week 9 was no longer significant (**Fig. 3D, left panel**), and while there was a modest but consistent decrease in pathology at week 9, granuloma volume and fibrosis became indistinguishable between WT and DKO + αIL-25-treated mice (**Figs. 3A-C**). Indeed, both groups of mice displayed a striking increase in IL-13-dependent fibrosis by week 12 as determined by both hydroxyproline assay (**Fig. 3B**) and picrosirius red staining (**Fig. 3E**). In addition, while the frequency of IL-13-producing ILC2s was lower in the MLN at week 9 (**Fig. 3D**), a marked increase in IL-4- and IL-13-producing $CD4^+$ T cells was observed at the same time point in the granulomatous livers of the DKO + αIL-25-treated mice (**Fig. 3F**). Antigen-specific $CD4^+$ Th2 cell cytokine production likely compensated for the transient decrease in ILC2s, thus explaining the unimpaired development of IL-13-dependent fibrosis in triple deficient mice by week 12.

Disrupting TSLP, IL-25, and IL-33 signaling during acute granuloma formation

After considering the transient nature of immune control affected by disrupting the three mediators during *S. mansoni* infection, we hypothesized that the effect of blocking all three cytokines would be more apparent when applied to a more acute model where the cytokines are blocked from the onset of injury. Primary and secondary lung granuloma models were employed for these studies because they provide simple and short-term systems to dissect the importance of TSLP, IL-25, and IL-33 during both the sensitization and maintenance phases of a type 2 cytokine-driven inflammatory response (*31*). Groups were treated with either isotype control antibodies or with αTSLP, αIL-25□□□□□□αST2 (IL-33 receptor) monoclonal neutralizing antibodies for the entire length of the experiments. The pathological effects of TSLP, IL-25, and

207 IL-33 have been directly linked to the enhanced production of IL-4, IL-5, and IL-13 by
208 downstream target cells such as $CD4^+$ Th2 cells, ILC2s, and other innate lymphocytes (*33, 34*),
209 and much of the pathology that results from the persistent activation of type 2 immunity has been
210 attributed to IL-4/IL-13-mediated signaling through the IL-4 receptor (*35*). Therefore, we used
211 IL-4R Γ -deficient mice as positive controls.

212 Surprisingly, as observed in previous lung granuloma studies where TSLP, IL-25, and IL-
213 33 were targeted individually, the combined blockade of all three cytokines had no significant
214 impact on the volume of the lesions in mice undergoing either primary (**Fig. 4A**) or secondary
215 (**Fig. 4B**) granuloma formation. In marked contrast, the lesions in IL-4Rα$^{-/-}$ mice were about
216 50% smaller than those in isotype control treated mice (**Figs. 4A-B**). The triple blockade did
217 lead to a >80% reduction in the number of granuloma-associated eosinophils during primary
218 granuloma formation (**Fig. 4A, right panel and tissue sections**). Macrophages and primarily
219 lymphocytes comprised the granulomas in the absence of eosinophils. Nevertheless, the
220 eosinophil deficit in the triple blockade mice was completely corrected when the mice were
221 undergoing a secondary challenge (**Fig. 4B, right panel and tissue sections**). IL-4Rα$^{-/-}$ mice, in
222 contrast, displayed a near complete absence of eosinophils following both primary and secondary
223 challenges.

224 Although the effects of the triple blockade on egg-induced pathology were minimal, there
225 were notable changes in cytokine expression in the lungs. Triple blockade mice displayed
226 significant reductions in IL-4, IL-5, and IL-13 expression in the lung during primary granuloma
227 formation (**Fig. 4C**) and in IL-5 and IL-13 during secondary granuloma formation (**Fig. 4D**). It
228 is worth noting that while these measurements imply a significant reduction in the type 2
229 cytokines following triple blockade, they were expressed at significantly higher levels than that

in IL-4Rα–deficient mice. Interestingly, changes in expression of two eosinophilic chemokines, *Ccl5* and *Ccl11,* do not explain the eosinophil phenotype we observed. *Ccl5* and *Ccl11* were not affected by the triple blockade during the primary response (**Fig. 4C**) although both chemokines were reduced in the triple blockade mice during a secondary response (**Fig. 4D**). Rather, the pattern of *Il5* gene expression likely explains why granuloma eosinophilia is reduced by the triple blockade during primary granuloma formation and is restored during secondary granuloma formation. The reduced *Il5* expression in triple blockade mice during primary granuloma formation was on par with expression observed in IL-4Rα -deficient mice. During secondary granuloma formation, *Il5* expression was reduced by the triple blockade, but it was still expressed at significantly higher levels than in IL-4Rα –deficient mice.

As seen in many type 2 cytokine-driven diseases, we observed increased *Il33*, *Tslp*, and *Il25* gene expression in the lungs of wild-type mice in the primary granuloma model (**Fig. 4C**). While gene expression of *Il33* and *Tslp* increased in the lungs of mice undergoing secondary granuloma formation, *Il25* was expressed at baseline levels during the secondary response (**Fig. 4D**). The increase was IL-4Rα-dependent as *Il33* and *Tslp* expression diminished to baseline levels in IL-4Rα$^{-/-}$ mice. We hypothesize the low alarmin expression in IL-4Rα$^{-/-}$ mice is due to decreased inflammation-driven injury in these mice.

Together, our studies with *S. mansoni* demonstrated that TSLP, IL-25, and IL-33 play redundant roles in the maintenance of chronic type 2 immunity. More importantly, targeting all three cytokines simultaneously from the initiation of primary or secondary granuloma formation reduced type 2 cytokine production but offered little protection from egg-induced pathology.

Efficacy of TSLP, IL-25, and ST2 blockade on established chronic allergy

With evidence that the combined blockade of TSLP, IL-25, and IL-33 signaling had a significant impact on type 2 cytokine expression, we hypothesized that the triple blockade might ameliorate type 2-mediated pathology in a different disease model. We sought to investigate a model that primarily targets epithelial cells, the predominant source of TSLP, IL-25, and IL-33, to determine whether the maintenance of type 2 immunity induced via mucosal epithelial injury was more dependent on the targeted cytokines. We chose to test the effects of administering single, double, and triple mAb blockades to a model of house dust mite (HDM)-induced allergic inflammation entering its chronic stage. Genes for all three alarmins are expressed at steady state in the lung, and HDM induces expression of each of the alarmins with complementary kinetics **(Fig. S2)**. *Il33* was upregulated acutely and at chronic stages of allergic disease. *Tslp* was only upregulated in the initial hours after first HDM exposure, and *Il25* was upregulated only at chronic time-points. BALB/c mice were chronically challenged i.n. with HDM on days 0, 7, and 14 and then received eight additional doses spread over a total of 45 days. Beginning three weeks after the initiation of the allergic response, separate groups of HDM-treated mice were administered doses of anti-ST2, anti-TSLP, anti-IL-25 every 3 to 4 days in various combinations to achieve single, double, or triple blockades. Additional control groups received either saline or isotype control antibodies with or without HDM. On day 46, all mice were analyzed. As expected, in the lungs of isotype-treated control mice, chronic HDM exposure resulted in a marked increase in inflammatory cells in the lung (**Fig. 5A**) and nearly a two-fold increase in collagen content (**Fig. 5B**), confirming extensive lung remodeling and fibrosis. Surprisingly however, none of the single, double, or triple blockade combinations led to a significant decrease in inflammation or fibrosis in the sensitized mice. When the triple blockade mice were analyzed more closely, we also observed little to no change in the type 2 cytokine

response in the lung (**Fig. 5C**), and the total number of leukocytes in the BAL and lung appeared indistinguishable between the triple blockade and isotype control treated mice (**Fig. 5D**). We did, however, observe a significant decrease in the percentage of eosinophils in the lung but not in the BAL (**Fig. 5E**).

Disrupting TSLP, IL-33, and IL-25 signaling during initiation and maintenance of type 2-driven chronic allergy

The failure of the triple blockade to protect against type 2-driven pathology when applied to established allergy further suggested that TSLP, IL-25, and IL-33 are not critical for the maintenance of chronic type 2 driven allergic lung inflammation. To test whether disrupting signaling of the three cytokines during the initiation of type 2 cytokine-driven allergic lung inflammation provides a benefit, in a final series of experiments, IL-33/TSLP DKO mice were treated with anti-IL-25 during the entire course of chronic HDM exposure. Here, the deficient mice displayed marked and significant decreases in fibrosis when compared with control HDM mice on day 46 (**Fig. 6A**). Although peribronchial and perivascular inflammation in the lung was similar in both groups, we observed a marked decrease in endarteritis and mucus staining in the lumen of the deficient mice (**Fig. 6B**). In addition, the total number of BAL cells (**Fig. 6C**), and the number of eosinophils in the BAL (**Fig. 6D**) and lung (**Fig. 6E**) were reduced. The decrease in inflammatory eosinophils was also accompanied by a marked and highly significant reduction in IL-4, IL-5, and IL-13 production in the lung (**Fig. 6F**) and IL-13 and IL-5 were also significantly decreased in the BAL (**Fig. 6G**). We observed similar results using anti-ST2, anti-TSLP, anti-IL-25 neutralizing antibodies in wild-type mice during the entire course of chronic HDM exposure **(Fig. S3)**.

Discussion

Although TSLP, IL-25, and IL-33 have each been identified as important initiators of type 2 immunity, their role in the maintenance of progressive type 2 disease was much less clear. In the present study, using chronic models of helminth infection and type 2 cytokine-driven lung inflammation, we found that tri-functional targeting of TSLP, IL-25, and IL-33 was more efficacious than blocking any one of the mediators alone. This conclusion is strengthened since we made the observations using mice on both C57BL/6 and BALB/c backgrounds. In a schistosome lung granuloma model or during chronic *S. mansoni* infection in the liver, selective ablation of TSLP, IL-25, or IL-33/ST2 had little to no impact on the development of IL-4/IL-13-dependent inflammation or fibrosis. Nevertheless, we observed modest albeit significant reductions in egg-induced inflammation in the liver when signaling of all three mediators was disrupted simultaneously. The reduction in inflammation in the schistosome infection model was also accompanied by a small yet significant decrease in the number of granuloma-associated eosinophils, a 25-30% decrease in hepatic fibrosis, and a significant reduction in the number of IL-13-producing ILC2s in the mesenteric lymph nodes. The deficient mice also displayed reduced expression of IL-5 and IL-13 during primary and secondary granuloma formation in the lung. Furthermore, when signaling of all three mediators was disrupted in a model of chronic HDM-induced allergic lung inflammation, inflammation, mucus production, and lung remodeling were decreased. Together, these studies revealed redundant roles for TSLP, IL-25, and IL-33 in the maintenance of these type 2-associated pathologies and suggest that aggressive

tri-functional targeting of these mediators may more effectively ameliorate progressive type 2-driven disease.

Previous studies identified critical roles for TSLP, IL-25, and IL-33 in type 2 immunity to some helminth parasites (*36-43*). However, the majority of these studies have focused on *Nippostrongylus brasiliensis* infection, in which expulsion of the nematode parasite is delayed or accelerated by relatively minor changes in type 2 immunity. Our initial studies focused on the schistosome lung granuloma and *S. mansoni* infection models because these models provide robust systems to dissect the role of TSLP, IL-25, and IL-33 during both the initiation and maintenance phases of type 2-driven inflammation (*31*). As reported previously with TSLP (*26*), we observed little to no role for IL-25 or IL-33 in IL-4/IL-13-dependent granuloma formation in the lung. A recent study found modest decreases in acute inflammation in the absence of IL-25 (*44*), but in our studies, IL-25 or IL-33 deficiency alone had no discernable impact on the development of type 2 immunity or type 2-dependent pathology, even during the initiation of a primary granulomatous response. A similar outcome was observed in the liver following acute and chronic infection with *S. mansoni*, suggesting that TSLP, IL-25, and IL-33 were either not required or were possibly playing redundant roles (*26, 28, 38, 45*). Importantly, although we found little to no role for TSLP, IL-25, or IL-33/ST2 when each mediator was ablated individually, we observed significant reductions in type 2 inflammation and fibrosis in the liver when all three mediators were targeted simultaneously, confirming their overlapping activities in response to significant damage during acute schistosomiasis. It is possible the degree of damage from parasites and other environmental triggers may impact the redundancy of the alarmins. Also, schistosome egg antigens have been identified that are capable of directly activating type 2 responses by modulating dendritic cell function(*46, 47*). Basophil- and autocrine T cell-derived

IL-4 may also be sufficient to initiate and maintain type 2 responses(*48, 49*). Therefore, alarmins may not be critical to the activation or maintenance of all type-2 cytokine driven inflammatory responses.

The type 2 response is a critical driver of wound repair pathways (*1*). However when type 2 cytokine production persists or becomes dysregulated, it can lead to the development of pathological fibrosis (*3*). Consequently, because of their type 2-inducing activity, there has been a great deal of interest in understanding the roles of TSLP, IL-25, and IL-33 in progressive fibrosis, with numerous studies identifying increased production of these cytokines in various fibrotic diseases (*50-54*). Many recent studies have shown that when overexpressed in mice, TSLP, IL-25, and IL-33 induce fibrosis in multiple tissues. For example, IL-25 was shown to promote lung remodeling in a model of house dust mite induced allergic airway disease and indirectly induced pulmonary fibrosis by stimulating the production of IL-13 from ILC2s (*44, 54*). Transgenic overexpression of IL-33 has also been shown to promote IL-13-dependent cutaneous fibrosis (*55*), ILC2-mediated hepatic fibrosis (*32*), and bleomycin-induced pulmonary fibrosis in mice (*56*). Transgene-induced expression of TSLP has also been shown to induce pulmonary fibrosis in the lung by upregulating type 2 cytokine expression (*52*). Nevertheless, evidence that these epithelial-derived alarmins are critical to the development of Th2-associated fibrosis in a natural model of fibrosis was lacking prior to this study. Our studies with the schistosome lung granuloma and infection models show quite unequivocally that IL-13-dependent fibrosis can develop in the lung and liver independently of TSLP, IL-25, and IL-33. We did, however, observe a significant decrease in fibrosis when all three mediators were targeted simultaneously, with the reduction in fibrosis associated with a significant decrease in IL-13 producing ILC2s. Surprisingly, at more chronic time points following infection with *S.*

367 *mansoni* the early reduction in fibrosis and ILC2 activity appeared to be compensated for by an
368 increased $CD4^+$ T cell-derived IL-13 response, suggesting that TSLP, IL-25, IL-33 and ILC2s
369 may not be critical to the maintenance of established and progressive fibrosis once the adaptive
370 immune response has taken over. The relative involvement of an adaptive antigen-specific
371 response may therefore be important in determining the relative contribution of these innate
372 pathways to chronic disease. Regardless, these data further emphasize the potential benefit of
373 early combinatorial targeting of TSLP, IL-25, and IL-33 in the treatment of type 2-driven
374 disease.

375 Because epithelial cells are a major source of TSLP, IL-25, and IL-33 and schistosome
376 eggs primarily damage the endothelium, it is possible that these cytokines are less important to
377 the development of type 2 pathology in schistosomiasis. Therefore, in a final series of
378 experiments, we utilized a chronic model of HDM-induced allergic lung inflammation to explore
379 the combined roles of TSLP, IL-25, and IL-33 in a disease where the epithelium is the primary
380 target. Here, in contrast to the lung granuloma studies in which a mAb triple blockade
381 administered from initial egg challenge had little impact on type 2 pathology, disrupting TSLP,
382 IL-25, and IL-33 signaling from first allergen exposure had a significant suppressive effect on
383 the development of fibrosis, endarteritis, and mucus deposition in the lumen. The number of
384 inflammatory cells in the BAL was also reduced, as were the number of eosinophils in the BAL
385 and lung, with the reduction in eosinophils consistent with a recent study exploring the roles of
386 TSLP, IL-25, and IL-33 in a model of chitin-induced lung inflammation (*57*). We also observed
387 marked and highly significant reductions in IL-5 and IL-13 production in the lung and BAL
388 fluid. When the combined mAb blockade of TSLP, IL-25, and IL-33 was applied to a model of
389 established allergic lung inflammation, the marked protective effects were almost completely

lost, however, suggesting that TSLP, IL-33, and IL-25 are either not required for the maintenance of an established antigen-specific type 2 response or that earlier intervention with TSLP, IL-33, and IL-25 antagonists is needed.

Although TSLP, IL-33, and IL-25 were all initially identified as critical drivers of type 2 immunity (*10, 12, 50*), several subsequent studies have illustrated that type 2 immunity can develop independently of these cytokines (*24-26, 28*). The results from our experiments suggest that much of the data in the latter studies are likely explained by the overlapping activities of TSLP, IL-33, and IL-25. Our data also suggest the three alarmins may be dispensable for the maintenance of type 2 immunity and chronic type 2-associated pathology because continued exposure to complex antigens like schistosome eggs or house dust mite allergen generates a potent and sustained adaptive $CD4^+$ type 2 response that can supplant the requirement for alarmins and innate lymphocytes. A recent double-blind, placebo-controlled study of AMG 157, a neutralizing anti-human TSLP mAb, showed that TSLP blockade could reduce allergen-induced bronchoconstriction and eosinophilia (*58*). Whether targeting TSLP alone would show clinical benefit in moderate-severe asthma, however, could not be discerned from this small study tested on allergic individuals with near-normal baseline lung function.

Differences in perturbations of epithelium and other stromal cells may dictate the relative contribution of the three alarmins, and further studies with different animal models of allergy (e.g. allergen dosing, variety, airway hypersensitivity) will be important before large-scale human studies are considered. The cost and time required for chronic models prevented us from testing all combinations of single, double, and triple blockades in every model. Notably, the triple blockade with mAbs from the start of allergic disease is effective, but its impact was not identical to congenital knockouts by all measures. Although all three antibodies were confirmed

to exhibit highly effective neutralizing activity, it is possible that incomplete target coverage with the antibodies might in part explain these differences as well as the minimal efficacy of treating mice with established allergic disease. It is also possible that intracrine alarmin signaling such as IL-33-mediated activation of NF-κB contributes to these small differences. In any case, antibody target coverage should be carefully evaluated in any future study in humans. Chronic human disease is likely maintained by a complex assortment of signals combined with sporadic exposure to specific antigen, and a better understanding of the hierarchy of these cues will help to clarify the relative contributions of TSLP, IL-33, and IL-25, as well as ILC2s. Our data suggest that a strategy that simultaneously suppresses more than one of these alarmins from the early phase of the disease may be required to effectively target type 2 cytokine-driven disease.

Materials and Methods

Study Design

Our primary objective was to investigate the effects of ablating IL-33, TSLP, and IL-25 signaling on chronic type 2 inflammation and fibrosis. To do this, we developed strategies to disrupt the signaling of the cytokines in mouse models of progressive type 2 immune-related pathology. No statistical methods were used to predetermine sample size. Group sample size was chosen using records of variance in past experiments, and variance is similar between groups being statistically compared. Samples or data points were excluded only in the case of a technical equipment or human error that caused a sample to be poorly controlled for. Mice or samples were randomly assigned to experimental groups or processing orders. Group allocation was blinded for all mouse work, when possible (e.g. administration of proteins, schistosomes, or

allergens, sample quantification and analysis, pathology scoring). The ARRIVE guidelines in the EQUATOR Network library were followed for this report.

Animals

The National Institute of Allergy and Infectious Diseases Division of Intramural Research Animal Care and Use Program, as part of the National Institutes of Health Intramural Research Program, approved all of the experimental procedures (protocol "LPD 16E"). The Program complies with all applicable provisions of the Animal Welfare Act (http://www.aphis.usda.gov/animal_welfare/downloads/awa/awa.pdf) and other federal statutes and regulations relating to animals. IL-33$^{-/-}$ and IL-33/TSLP double knockout mice on a C57BL/6 background were provided by Amgen Inc. C57BL/6, BALB/c, and IL-4Rα$^{-/-}$ mice were obtained from Taconic Farms Inc. IL-25$^{-/-}$ mice were obtained from Regeneron Pharmaceuticals, Inc. Male and female mice between the ages of 6 weeks and 12 weeks were used randomly to begin experimental models because of limited availability, and no sex-specific differences were observed. Groups in individual experiments were sex-matched and age-matched. All animals were housed under specific pathogen-free conditions at the National Institutes of Health in an American Association for the Accreditation of Laboratory Animal Care-approved facility.

Parasite infection

Mice were infected percutaneously via the tail with 35 cercaria from a Puerto Rican strain of *Schistosoma mansoni* (NMRI) obtained from infected *Biomphalaria glabrata* snails (Biomedical Research Institute). 35 cercaria infection in wild-type mice leads to substantial disease and liver

fibrosis but low mortality through the chronic phase of infection. Mice were perfused at the time of euthanasia to determine worm and tissue egg burdens as described previously (*59*).

Chronic house dust mite-induced allergy

Mice anesthetized with isoflurane were challenged intranasally with 200 µg of house dust mite (HDM) in 30µl saline on days 0, 7, and 14 followed by eight additional 50 µg doses in 30µl saline spread over a total of 45 days. Lungs were harvested on day 46.

Schistosome egg-induced lung granuloma models

For the primary lung granuloma model, 5000 live *S. mansoni* eggs (Biomedical Research Institute) in saline were injected intravenously into mice on day 0. Lungs were harvested on day 7 for analysis. For the secondary lung granuloma model, 5000 *S. mansoni* eggs were also injected intraperitoneally on day 0. Mice were injected intravenously with 5000 live eggs containing mature embryos again on day 14 before lungs were harvested on day 21.

Hydrodynamic delivery of IL-25

Mice were injected intravenously with 10µg of a mammalian expression plasmid coding for murine IL-25 in 2ml of warm saline (*60*).

Triple block of IL-33, TSLP, and IL-25 with monoclonal antibodies

Anti-mouse ST2 (*61*), anti-mouse TSLP (*38*), and anti-mouse IL-25 (*62*) monoclonal antibodies were generated and selected by Amgen Inc. after extensive *in vitro* and *in vivo* testing. Previously unpublished tests for the efficacy of anti-TSLP included a bone marrow-derived

dendritic cell bioassay measuring the inhibition of TSLP-induced CCL17/TARC production and an assay measuring inhibition of TSLP-induced proliferation of a pro-B cell line stably transduced with murine TSLP receptor. Neutralization of IL-33, TSLP, and IL-25 signaling was achieved by administering 250μg of these antibodies, respectively, via intraperitoneal injection twice-weekly. To properly control for the neutralizing antibodies, groups administered single and double blocks also received 250μg mouse IgG1 in the absence of anti-ST2 or anti-IL-25, and 250 g rat IgG1 in the absence of anti-TSLP.

Histopathology

Liver or lung tissue was fixed in Bouin-Hollande solution, embedded in paraffin for sectioning, and stained (Histopath of America) with Wright's Giemsa (*S. mansoni* models), hematoxylin and eosin, or Masson's trichrome (allergy model) for analysis of inflammation, picrosirius red or Masson's trichrome for fibrosis analysis, or Periodic acid-Schiff (PAS) stain for analysis of mucus production. A scale of 1 to 4 (4 being the highest) was used for scoring. A blinded pathologist measured the diameter of approximately 30 granulomas and quantified granulomatous eosinophils in Giemsa-stained sections of each sample with granulomatous pathology. Images were scanned with an Aperio ScanScope (Leica Biosystems).

Fibrosis assay

Hydroxyproline was measured as a surrogate for collagen content. A known weight of liver or lung tissue was hydrolyzed in 6 N HCl at 110^{o}C for 18 h and then neutralized in 10 N NaOH before colorization. A standard curve comprised of dilutions of 1mM hydroxyproline (Sigma-Aldrich) (*63*).

Leukocyte isolation for intracellular cytokine staining and eosinophil identification

About 200 mg of lung or liver tissue was ground into a single-cell suspension through a 100-µm nylon mesh. Leukocytes were separated on a 40% Percoll (Sigma-Aldrich) gradient (2000 rpm for 15 min) and treated for 2 min with 1 ml ACK (ammonium chloride–potassium bicarbonate) lysis buffer to lyse erythrocytes. After 3 hours of stimulation with phorbol 12-myristate 13-acetate (PMA 10ng/ml), ionomycin (1µg/ml), and Brefeldin A (BFA, 10µg/ml), leukocytes were fixed and permeabilized for 30 minutes (Cytofix/Cytoperm buffer; BD Biosciences) and then stained for 30 minutes with antibodies for CD4 (Clone: RM4-5; eBioscience), IFN-γ (XMG1.2, eBioscience), IL-4 (11B11, eBioscience), IL-5 (TRFK5, BD Pharmingen), and IL-13 (eBio13A, eBioscience) diluted in the Permwash buffer (BD Biosciences). Unstimulated lung leukocyte aliquots were set aside and stained for 30 minutes with anti-SiglecF. Postive SiglecF staining and scatter profiling were used to identify eosinophils by flow cytometry. Leukocytes collected from bronchoalveolar lavage were isolated with ACK lysis buffer, stimulated, fixed, permeabilized, and stained as above. Expression of CD4, SiglecF, and the intracellular cytokines was analyzed with a BD FACSCanto II flow cytometer and FlowJo v.7.6 software (Tree Star).

Leukocyte isolation from liver and mesenteric lymph node for ILC2 staining

Liver or lymph node tissue was ground into a single-cell suspension through a 100-µm nylon mesh, and hepatic leukocytes required further separation using a 40% Percoll gradient and ACK lysis as described above. Leukocyte samples from both tissues were stimulated, fixed, and permeabilized as described above. Then they were stained for 30 minutes with antibodies for CD16/32 (Clone: 2.4G2, BDBiosciences), CD4 (RM4-5, eBioscience), IL-13 (eBio13A,

eBioscience), ST2 (DJ8, MD Biosciences), and ICOS (C398.4A, Biolegend) diluted in Permwash buffer (BD Biosciences). Expression of the surface markers and intracellular IL-13 was analyzed with a BD FACSCanto II flow cytometer and FlowJo v.7.6 software (Tree Star).

RNA isolation and quantitative real-time PCR

Lung or liver tissue was homogenized in TRIzol Reagent (Life Technologies) using Precellys 24 (Bertin Technologies). Total RNA was extracted from the homogenate by addition of chloroform followed by the recommendations of the MagMax-96 Total RNA Isolation Kit (Life Technologies). RNA was then reverse transcribed using SuperScript II Reverse Transcriptase (Life Technologies). Real-time RT-PCR was performed on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Quantities of mRNA expressed by a particular gene were determined using Power SYBR Green PCR Master Mix (Applied Biosystems), normalized to ribosomal protein, large, P2 (RPLP2) mRNA levels in each sample, and then articulated as a relative increase or decrease compared with mRNA levels expressed by the same gene in naive controls. Primers were designed using Primer Express software (version 2.0; Applied Biosystems). Forward and reverse primer sequences are listed in Table S1.

Bronchoalveolar lavage, cell differential determination, and ELISA

1 ml of ice-cold PBS supplemented with 5mM EDTA was injected through the trachea into the lungs and aspirated using a syringe. ~$1x10^5$ cells were spun for 5 mins with a Shandon Cytospin 3 centrifuge (Thermo Scientific) onto a slide before being fixed with methanol and stained with Diff-Quik (Boehringer) to identify leukocyte cell-types. Levels of IL-4, IL-5, and IL-13 in the

undiluted BAL were quantified using a Luminex-based multiplex assay according to manufacturer's protocol (EMDMillipore).

Statistical analysis

All data were analyzed with Prism (Version 5; GraphPad). Data sets were compared with a two-tailed t-test, and differences were considered significant if *P* values were less than 0.05. A Welch's correction was used when an F-test comparing variances had a *P* value of less than 0.05.

Supplementary Materials

Fig. S1. Alarmin gene expression in the liver.

Fig. S2. Kinetics of alarmin gene expression in chronic HDM model.

Fig. S3. Neutralizing all three alarmins with mAbs during initiation and maintenance of type 2-driven allergy reduces inflammation and fibrosis.

Table S1. qPCR Primer Sequences.

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Author contributions: KMV TRR MRC DES TAW conceived and designed the experiments; KMV TRR AWC LAB KMH RWT SW performed the experiments; KMV TRR LAB LB KMH KNK MRC DES TAW analyzed the data; KMH analyzed the statistics; AWC ALB MRC DES contributed reagents/materials/analysis tools; KMV TAW wrote the paper.

Competing interests: ALB, MRC, DES work for a for-profit company.

Data and materials availability: Genes of interest can be accessed in NCBI's GenBank with the following codes: *Rplp2:* NM_026020, *Il4:* NM_021283, *Il5:* NM_010558, *Il13:* NM_008355, *Ifnγ:* NM_008337, *Il25:* NM_080729, *Il13:* NM_008355, *Ccl5:* NM_013653, *Ccl11:* NM_011330, *Il33:* NM_001164724, *Tslp:* NM_021367.

Figures Legends

Figure 1. Ablating IL-25 offers no protection against type 2-mediated pathology.

A. Quantitative PCR analysis of gene expression in lung and liver tissue from wild type C57BL/6 mice seven days after hydrodynamic injection of IL-25 (n = 5 mice) or PBS (n = 2). B. Histopathology analysis of livers from wild type and IL-13R Γ 1$^{-/-}$ mice seven days after *S. mansoni* egg exposure and 8 days after hydrodynamic injection of IL-25 or PBS (n = 12-15 per group; pooled from two independent experiments; scale bars=50μm). C. Cytokine quantification from bronchoalveor lavage fluid (BALF) of mice in B (n = 4-5 per group). D. Histopathology analysis of lungs from IL-25$^{-/-}$ mice and littermate controls 7 days after challenge with *S. mansoni* eggs with (2^{o}) or without priming (1^{o}) with 5000 *S. mansoni* eggs 14 days prior to

challenge (granuloma volume 1°: n = 18-23 per genotype pooled from three experiments; granuloma volume 2°: n = 9-10 per genotype pooled from two experiments; eosinophils: n = 5 per genotype). E. Histopathology analysis and fibrosis quantificiation of livers of IL-25$^{-/-}$ mice and littermate controls 12 weeks after infection with *S. mansoni* cercariae (n = 9 per genotype). A Student's t-test was used to measure all P values, and P>0.05 except where reported. Error bars represent standard error of the mean and each data point represents a value for an individual mouse. Data are representative of two independent experiments unless otherwise noted.

Figure 2. Ablating IL-33 offers no protection against type 2-mediated pathology.

A. Fibrosis quantification and histopathology analysis of lungs from wild type C57BL/6 and IL-33$^{-/-}$ mice 7 days after challenge with *S. mansoni* eggs (n = 7-10 per genotype). B. Fibrosis quantification and histopathology analysis of lungs of the same mouse strains 21 days after priming with *S. mansoni* eggs and 7 days after challenge with eggs (n = 10 per genotype). C. Fibrosis quantification of livers from the same mouse strains infected with *S. mansoni* cercariae (n = 7-10 per genotype). D. Micrographs of representative liver tissue sections of mice in C collected 9 weeks after infection and stained with picrosirius red (scale bar=100μm). E. Histopathology analysis of livers from the mice in C (n = 7-10 per genotype). F. Intracellular cytokine analysis of lymphocytes isolated from livers of mice in C nine weeks after infection measured by flow cytometry (n = 8 per genotype). A Student's t-test was used to measure all P values, and P>0.05 except where reported. Data are representative of two independent experiments for each of the models.

Figure 3. Disruption of all three mediators simultaneously has a transient effect on Th2 pathology driven by *S. mansoni*.

A. Granuloma measurement (n = 14-19 per group pooled from two independent experiments) from livers of *S. mansoni*-infected wild type C57BL/6 mice administered isotype control antibody and IL-33/TSLP double knockout (DKO) mice administered anti-IL-25. B. Fibrosis quantification from livers of infected mice (n = 7-10 per group). C. Quantification of granuloma eosinophils from livers of infected mice (n = 7-9 per group). D. Quantification of $CD4^{-}IL\text{-}13^{-}ST2^{+}ICOS^{+}$ leukocytes from the mesenteric lymph nodes (MLNs; n = 7 per group) and livers (week 9: n = 7-8 per group; week 12: n = 14-15 per group pooled from two independent experiments) of infected mice by flow cytometry. E. Micrographs of representative liver tissue sections of mice 12 weeks after infection and stained with picrosirius red (scale bar=500μm). F. Intracellular cytokine analysis of liver lymphocytes of infected mice by flow cytometry (n = 14-17 per genotype pooled from two experiments). A Student's t-test was used to measure all P values, and $P>0.05$ except where reported. Data are representative of two independent experiments.

Figure 4. Combined TSLP, IL-25, and ST2 mAb blockade during granuloma generation diminishes type 2 immunity but not pathology.

A. Histopathology analysis of wild type BALB/c and $IL\text{-}4R^{-/-}$ mice seven days after injection with *S. mansoni* eggs. Wild type egg-injected mice were either intraperitoneally (IP) administered anti-ST2, anti-TSLP, and anti-IL-25, or corresponding isotype control antibodies (n = 8-9 per group). Micrographs are of representative lung sections stained with Masson's trichrome (scale bars = 50 μm). C. Quantification of gene expression in lung tissue from mice in

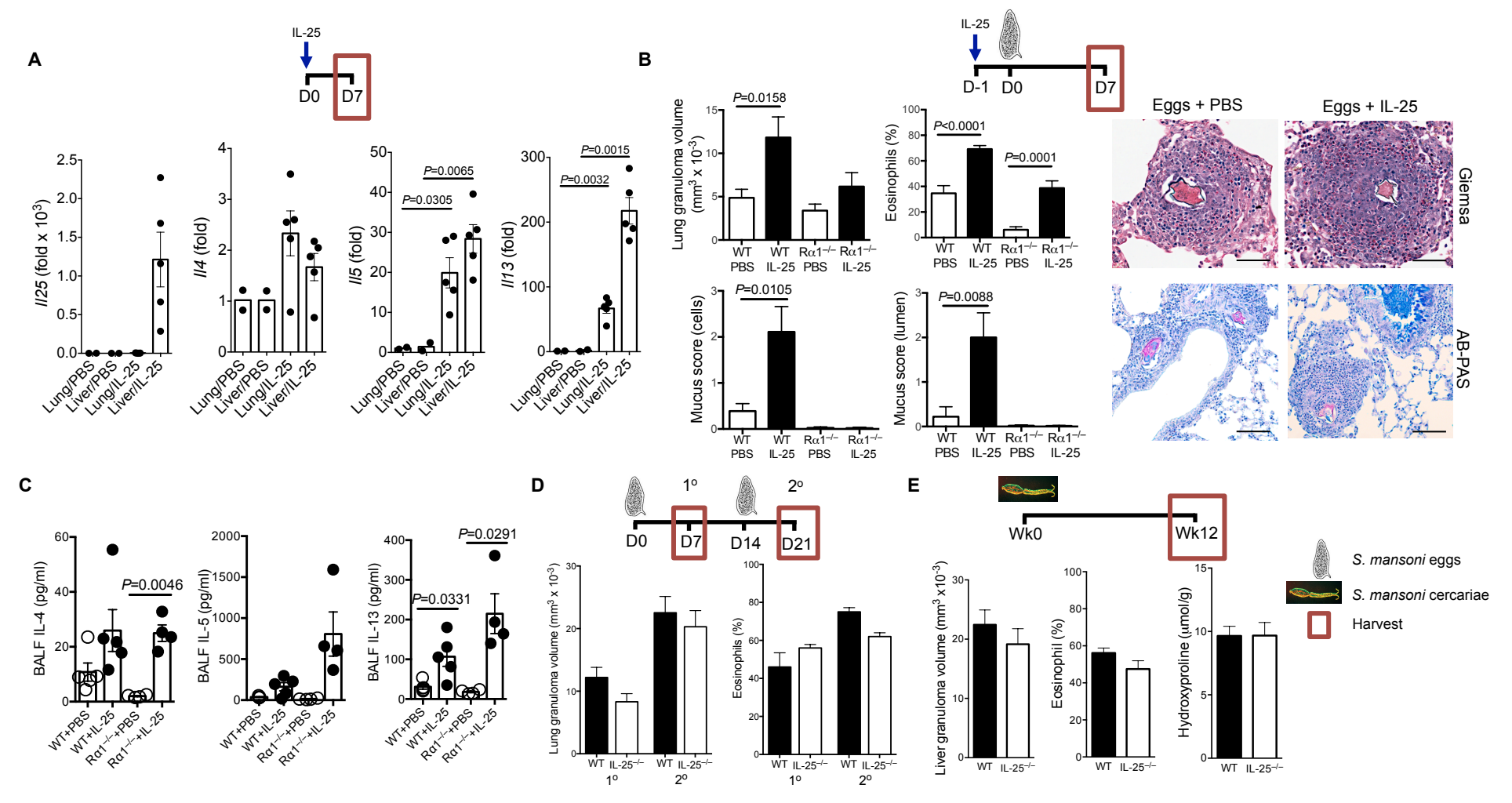
A assayed by qPCR and shown relative to expression in lungs of naïve BALB/c mice (n = 3). B. Histopathology analysis of wild type BALB/c and IL-4R$^{-/-}$ mice seven days after injection with *S. mansoni* eggs and 21 days after priming with *S. mansoni* eggs (Isotypes IP, Triple block IP: n = 8-9 per group; IL-4R$^{-/-}$: n = 5). Wild type egg-injected mice were either intraperitoneally administered anti-ST2, anti-TSLP, and anti-IL-25, or corresponding isotype control antibodies for all three weeks. Micrographs are of representative lung sections stained with Masson's trichrome (scale bars = 50 μm). D. Quantification of gene expression in lung tissue from mice in B assayed by qPCR and compared to a different group of naïve BALB/c controls (n = 3). A Student's t-test was used to measure all P values, and P>0.05 except where reported. Data are representative of two independent experiments.

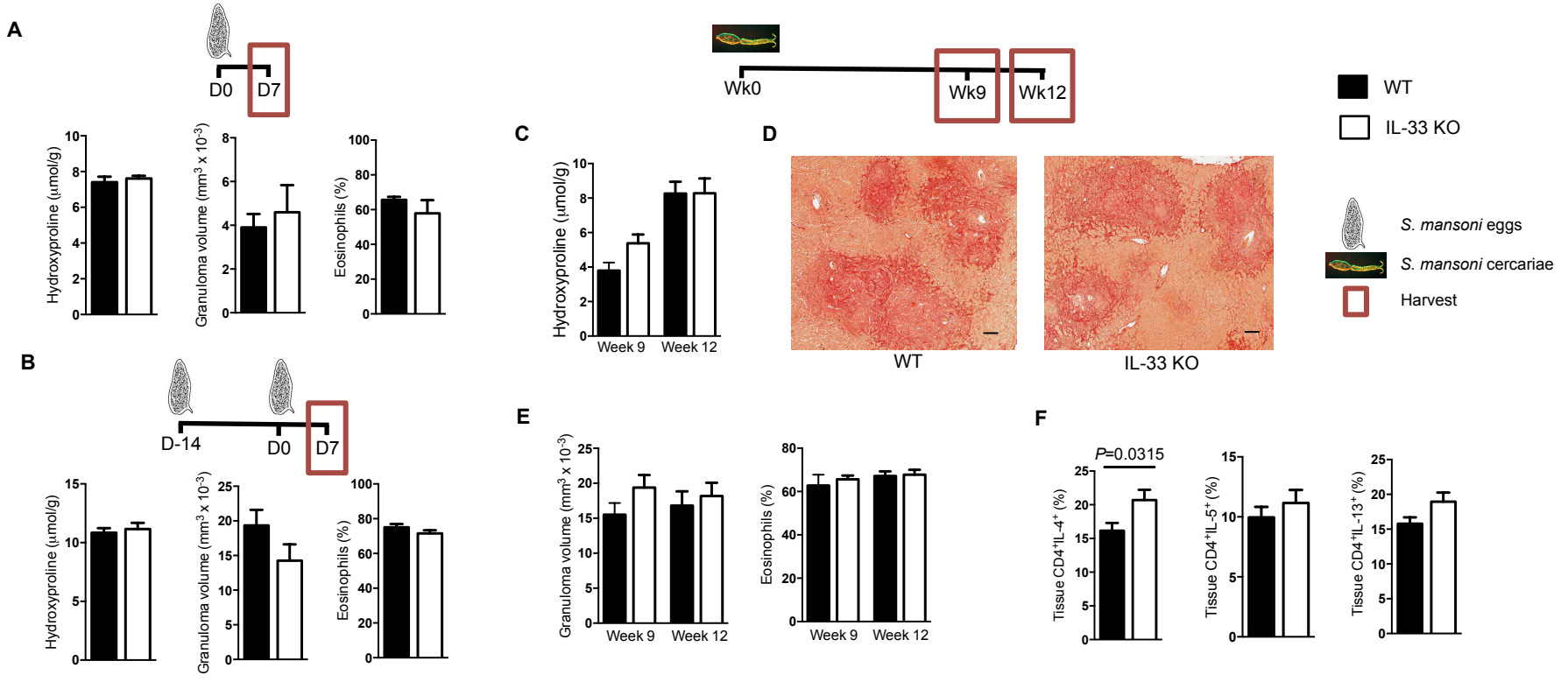
Figure 5. Efficacy of TSLP, IL-25, and ST2 mAb blockade on established chronic allergy. Wild type BALB/c mice were sensitized and challenged intranasally with house dust mite (HDM), and starting on day 21, anti-ST2, anti-TSLP, and/or anti-IL-25 were administered in various combinations to different groups to achieve single, double, or triple blocks. Additional control groups received only isotype control antibodies with or without HDM. To properly control for the triple blockade group, groups administered single and double blocks also received IgG1 in the absence of anti-ST2 or anti-IL-25, and rat IgG1 in the absence of anti-TSLP. All mice were analyzed on day 46. A. Histopathology analysis of lung sections stained with Masson's trichrome and scored for peribronchial and perivascular inflammation (n = 6-10 per group pooled from two experiments). B. Quantification of fibrosis from lung tissue. C. Quantification of gene expression from lung tissue measured by qPCR. D. Quantification of leukocytes in the BALF and lung tissue. E. Quantification of eosinophils shown as a percentage

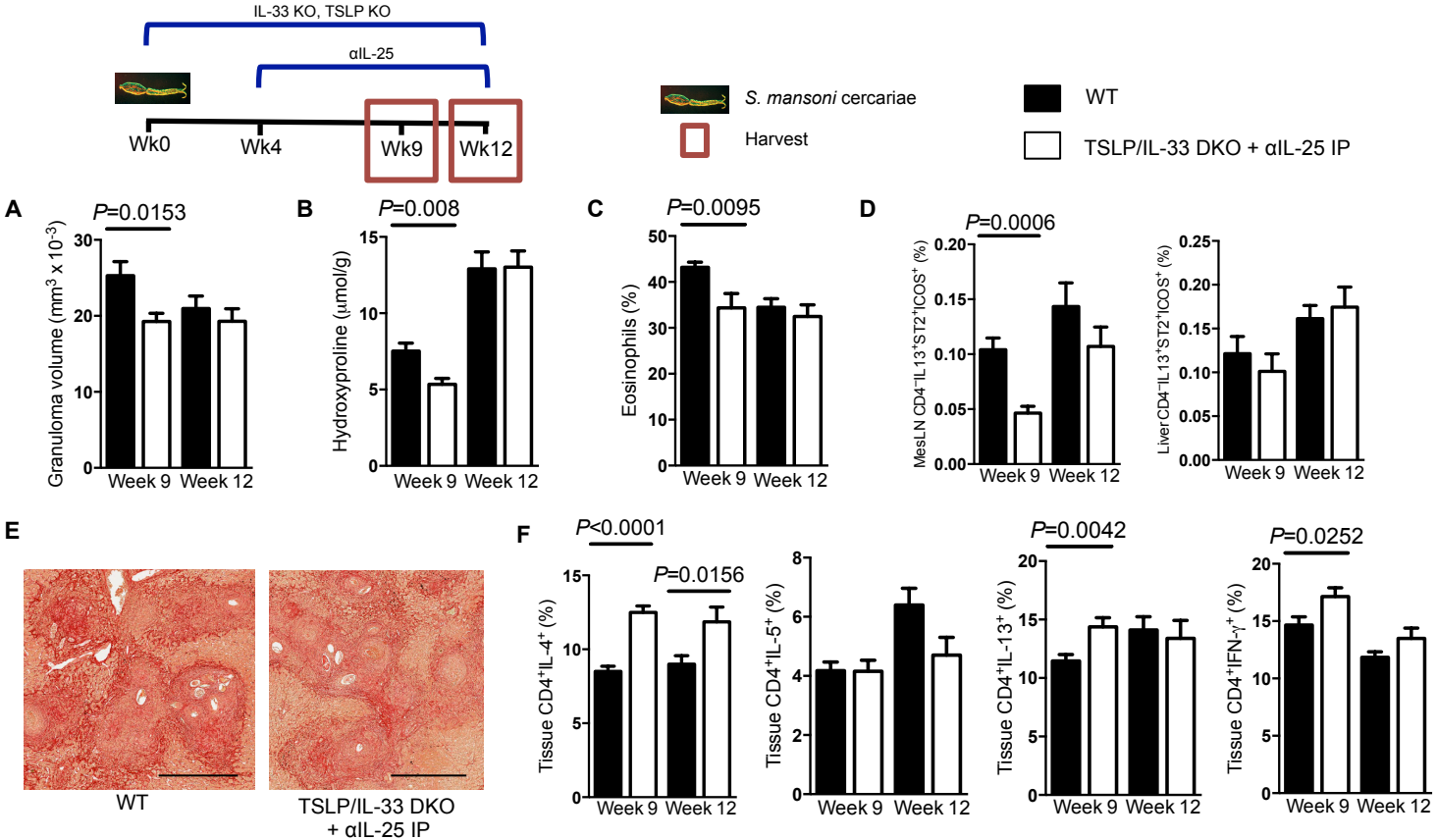
of total inflammatory cells in BALF and lung tissue. Student's t-test was used to measure all *P* values, and *P*>0.05 except where reported.

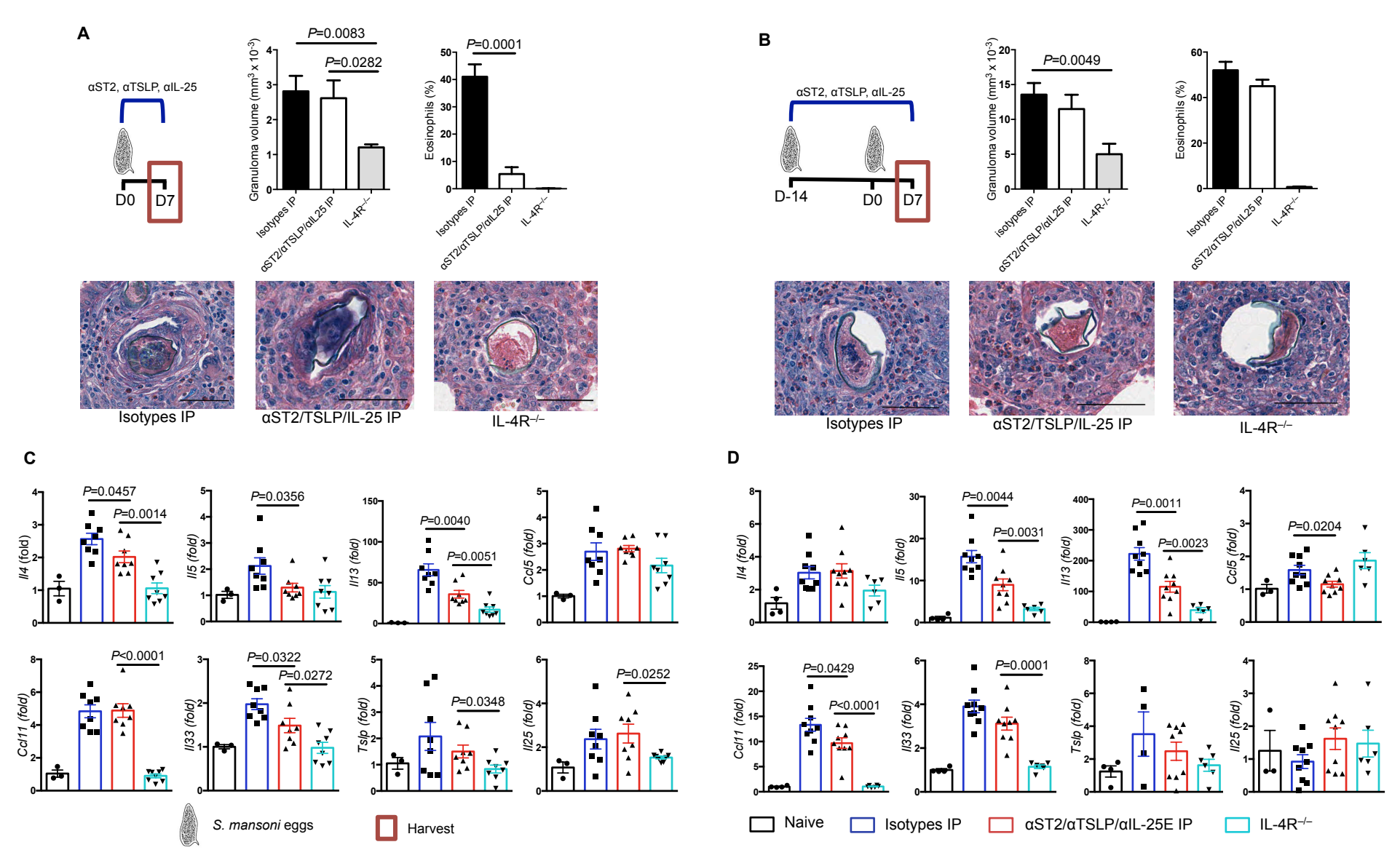
Figure 6. Disruption of all three mediators during initiation and maintenance of type 2-driven chronic allergy reduces inflammation and fibrosis.

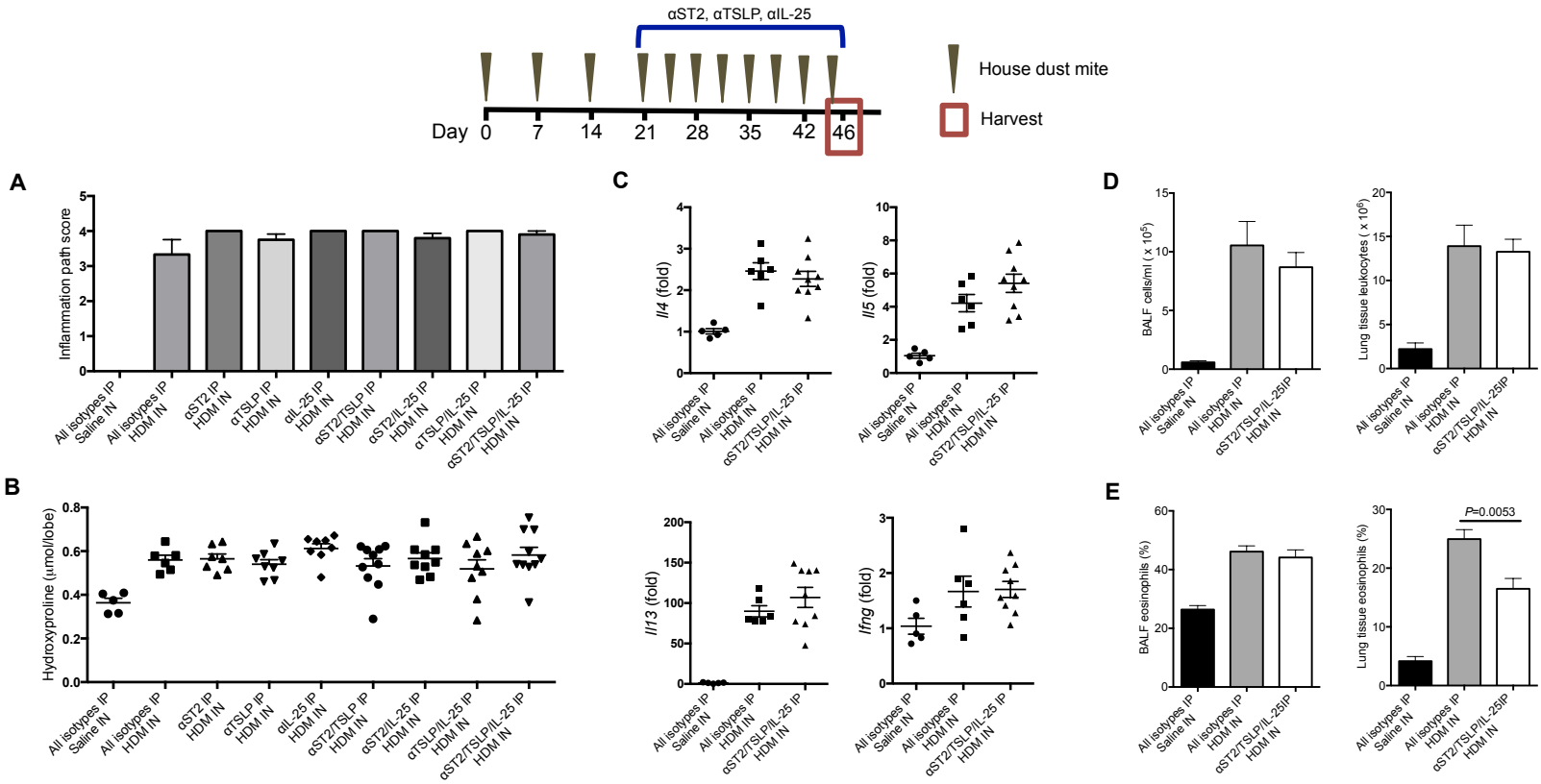
Wild type C57BL/6 and IL-33/TSLP DKO mice were sensitized and challenged intranasally with HDM over 45 days. DKO mice were IP administered αIL-25 (DKO+αIL-25/HDM), and HDM-treated wild-type C57BL/6 mice were IP administered an IgG1 isotype control (Isotype/HDM). A control group of C57BL/6 mice received intranasal saline instead of HDM and the isotype (Isotype/Saline). All mice were analyzed on day 46. A. Quantification of fibrosis from lung tissue (Isotype/Saline: $n = 5$; Isotype/HDM: $n = 9$; Triple block/Saline: $n = 8$). B. Histopathology analysis of lung sections stained with Masson's trichrome for scoring of inflammation and AB-PAS for mucus scoring. Micrographs are of representative lung sections stained with Masson's trichrome (scale bars = 50 μm). C. Quantification of leukocytes in the BALF. D. BALF leukocyte differential. E. Quantification of eosinophils in lung tissue. F. Intracellular cytokine quantification of lung tissue lymphocytes by flow cytometry. G. Intracellular cytokine quantification of BALF lymphocytes by flow cytometry. A Student's t-test was used to measure all *P* values, and *P*>0.05 except where reported. Data are representative of two independent experiments.

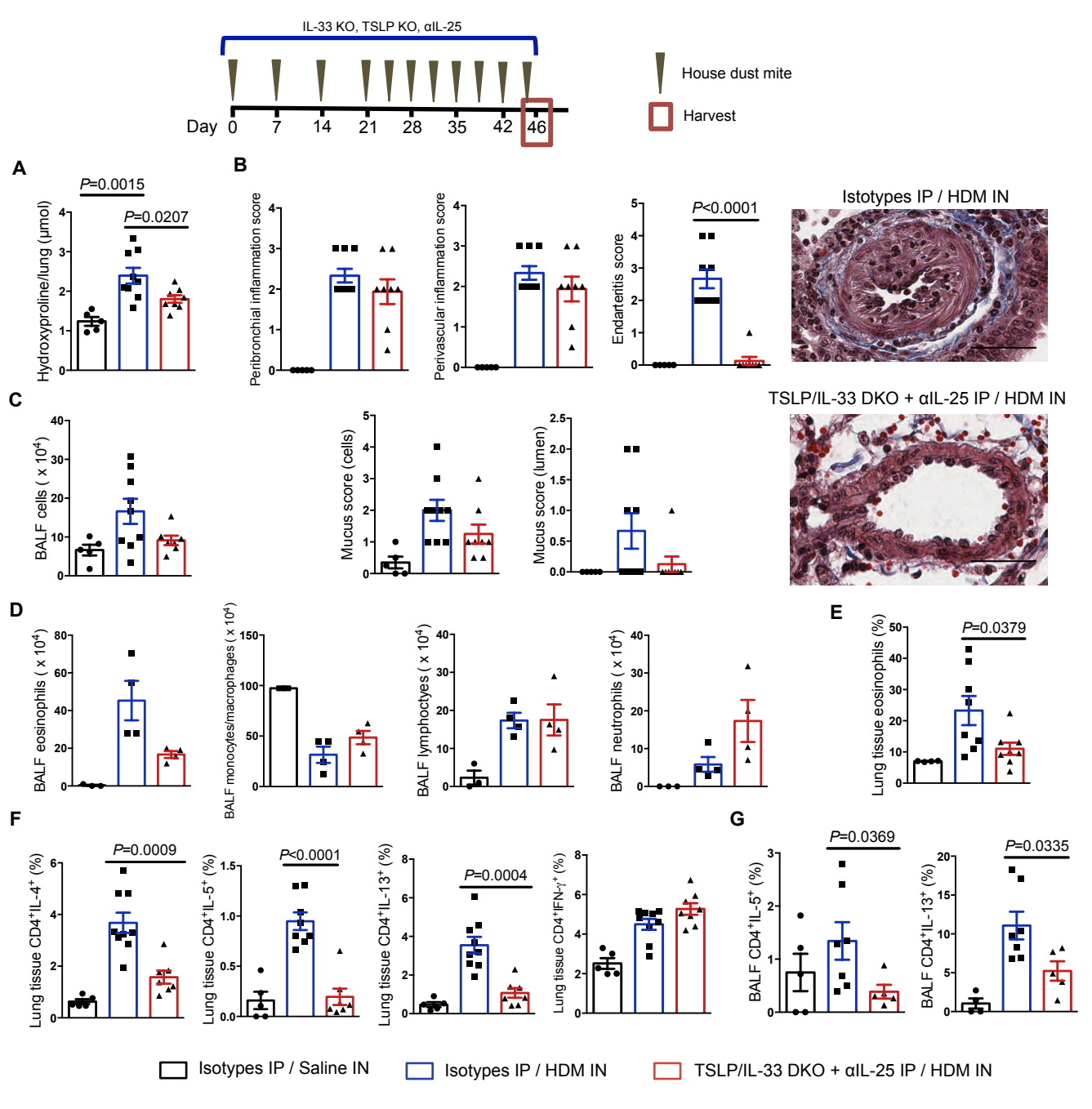

A
IL-25
D0
D7
Il25 (fold x 10^3)
Il4 (fold)
Il5 (fold)
Il13 (fold)
P=0.0305
P=0.0065
P=0.0032
P=0.0015
Lung/PBS
Liver/PBS
Lung/IL-25
Liver/IL-25
B
IL-25
D-1
D0
D7
Lung granuloma volume (mm3 x 10-3)
P=0.0158
Eosinophils (%)
P<0.0001
P=0.0001
Mucus score (cells)
P=0.0105
Mucus score (lumen)
P=0.0088
WT PBS
WT IL-25
Rα1−/− PBS
Rα1−/− IL-25
Eggs + PBS
Eggs + IL-25
Giemsa
AB-PAS
C
BALF IL-4 (pg/ml)
P=0.0046
BALF IL-5 (pg/ml)
BALF IL-13 (pg/ml)
P=0.0331
P=0.0291
WT+PBS
WT+IL-25
Rα1−/−+PBS
Rα1−/−+IL-25
D
1º
2º
D0
D7
D14
D21
Lung granuloma volume (mm3 x 10-3)
Eosinophils (%)
WT
IL-25−/−
E
Wk0
Wk12
Liver granuloma volume (mm3 x10-3)
Eosinophil (%)
Hydroxyproline (µmol/g)
S. mansoni eggs
S. mansoni cercariae
Harvest

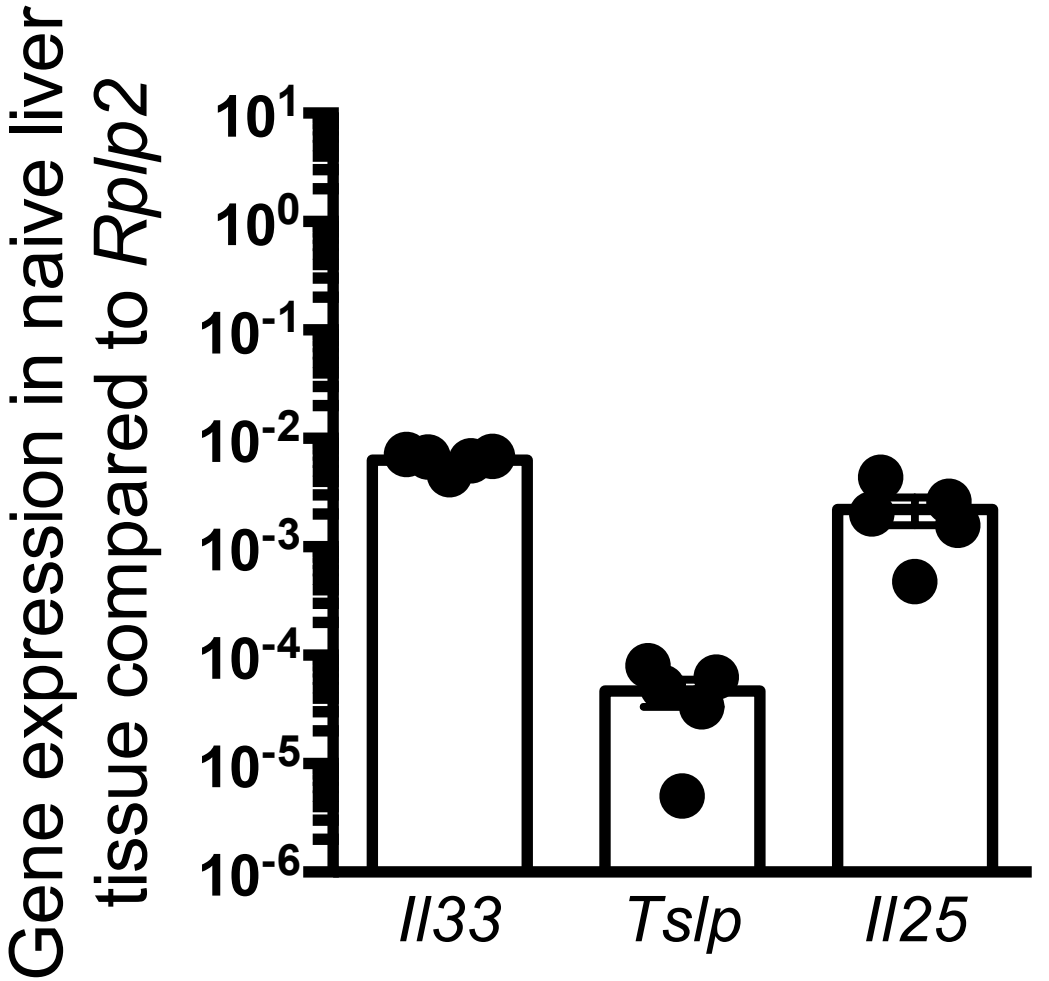

A
D0
D7
Hydroxyproline (µmol/g)
Granuloma volume (mm3 x 10-3)
Eosinophils (%)
B
D-14
D0
D7
Hydroxyproline (µmol/g)
Granuloma volume (mm3 x 10-3)
Eosinophils (%)
Wk0
Wk9
Wk12
C
Hydroxyproline (µmol/g)
Week 9
Week 12
D
WT
IL-33 KO
E
Granuloma volume (mm3 x 10-3)
Eosinophils (%)
Week 9
Week 12
F
P=0.0315
Tissue CD4+IL-4+ (%)
Tissue CD4+IL-5+ (%)
Tissue CD4+IL-13+ (%)
WT
IL-33 KO
S. mansoni eggs
S. mansoni cercariae
Harvest

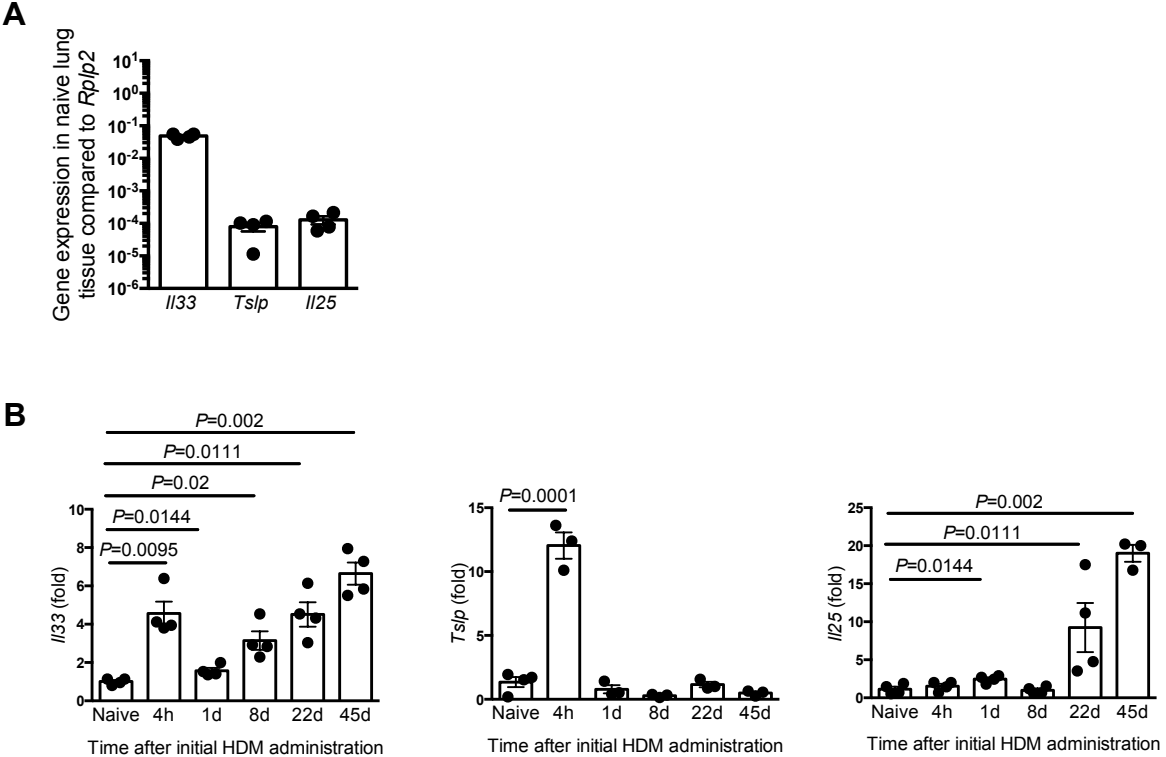

IL-33 KO, TSLP KO
αIL-25
Wk0
Wk4
Wk9
Wk12
S. mansoni cercariae
Harvest
WT
TSLP/IL-33 DKO + αIL-25 IP
A
P=0.0153
Granuloma volume (mm3 x 10-3)
Week 9
Week 12
B
P=0.008
Hydroxyproline (μmol/g)
C
P=0.0095
Eosinophils (%)
D
P=0.0006
MesLN CD4-IL13+ST2+ICOS+ (%)
Liver CD4-IL13+ST2+ICOS+ (%)
E
WT
TSLP/IL-33 DKO + αIL-25 IP
F
P<0.0001
P=0.0156
Tissue CD4+IL-4+ (%)
Tissue CD4+IL-5+ (%)
P=0.0042
Tissue CD4+IL-13+ (%)
P=0.0252
Tissue CD4+IFN-γ+ (%)

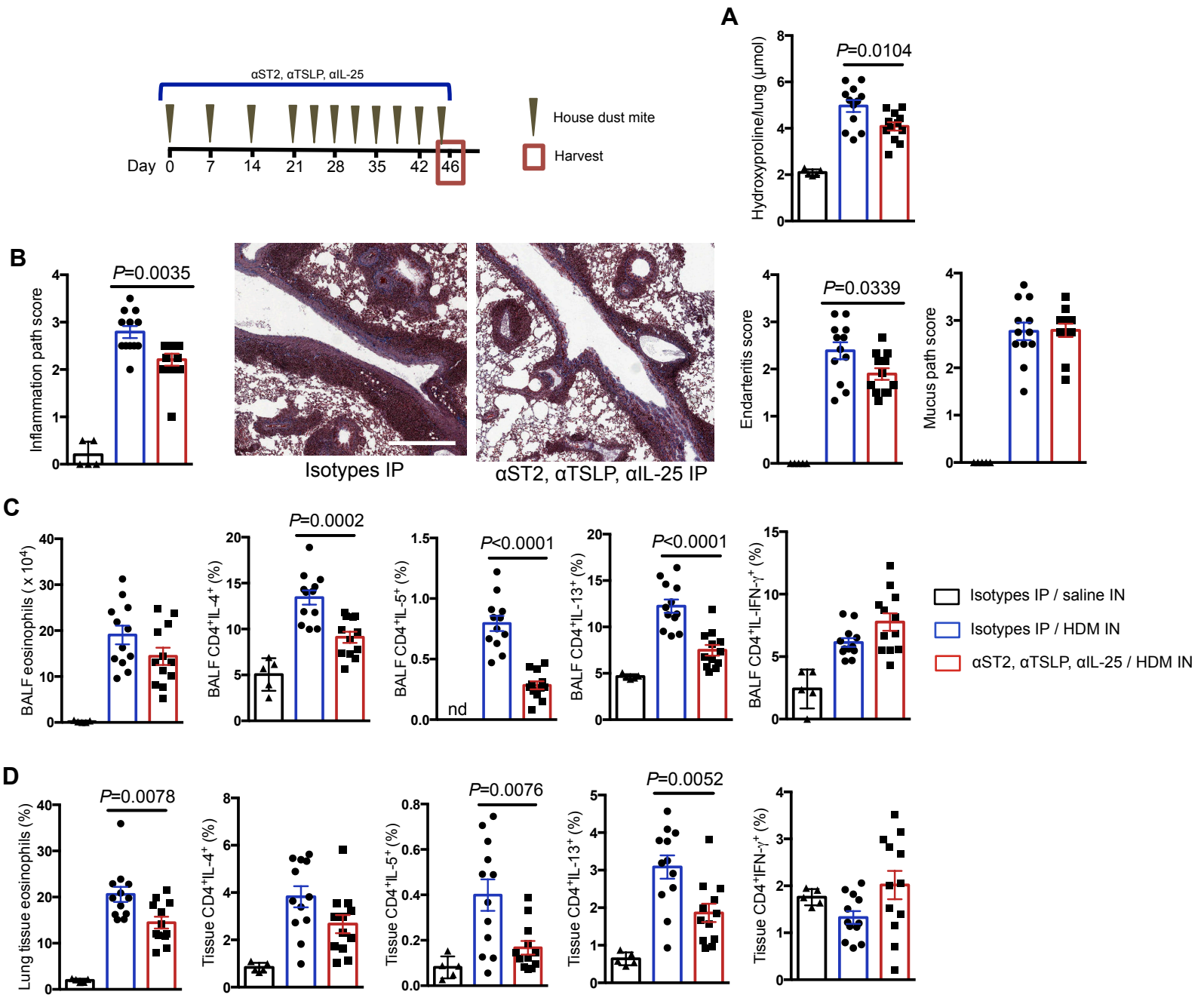

A
αST2, αTSLP, αIL-25
D0
D7
Granuloma volume (mm³ x 10⁻³)
P=0.0083
P=0.0282
Isotypes IP
αST2/αTSLP/αIL25 IP
IL-4R⁻/⁻
Eosinophils (%)
P=0.0001
Isotypes IP
αST2/αTSLP/αIL25 IP
IL-4R⁻/⁻
Isotypes IP
αST2/TSLP/IL-25 IP
IL-4R⁻/⁻
B
αST2, αTSLP, αIL-25
D-14
D0
D7
Granuloma volume (mm³ x 10⁻³)
P=0.0049
isotypes IP
αST2/αTSLP/αIL25 IP
IL-4R-/-
Eosinophils (%)
Isotypes IP
αST2/αTSLP/αIL25 IP
IL-4R⁻/⁻
Isotypes IP
αST2/TSLP/IL-25 IP
IL-4R⁻/⁻
C
Il4 (fold)
P=0.0457
P=0.0014
Il5 (fold)
P=0.0356
Il13 (fold)
P=0.0040
P=0.0051
Ccl5 (fold)
Ccl11 (fold)
P<0.0001
Il33 (fold)
P=0.0322
P=0.0272
Tslp (fold)
P=0.0348
Il25 (fold)
P=0.0252
S. mansoni eggs
Harvest
D
Il4 (fold)
Il5 (fold)
P=0.0044
P=0.0031
Il13 (fold)
P=0.0011
P=0.0023
Ccl5 (fold)
P=0.0204
Ccl11 (fold)
P=0.0429
P<0.0001
Il33 (fold)
P=0.0001
Tslp (fold)
Il25 (fold)
Naive
Isotypes IP
αST2/αTSLP/αIL-25E IP
IL-4R⁻/⁻


αST2, αTSLP, αIL-25
House dust mite
Harvest
Day 0 7 14 21 28 35 42 46
A
Inflammation path score
B
Hydroxyproline (μmol/lobe)
C
Il4 (fold)
Il5 (fold)
Il13 (fold)
Ifng (fold)
D
BALF cells/ml (x 10^5)
Lung tissue leukocytes (x 10^6)
E
BALF eosinophils (%)
Lung tissue eosinophils (%)
P=0.0053
All isotypes IP Saline IN
All isotypes IP HDM IN
αST2 IP HDM IN
αTSLP IP HDM IN
αIL-25 IP HDM IN
αST2/TSLP IP HDM IN
αST2/IL-25 IP HDM IN
αTSLP/IL-25 IP HDM IN
αST2/TSLP/IL-25 IP HDM IN


IL-33 KO, TSLP KO, αIL-25
Day 0 7 14 21 28 35 42 46
House dust mite
Harvest
A
P=0.0015
P=0.0207
Hydroxyproline/lung (μmol)
B
Peribronchial inflammation score
Perivascular inflammation score
Endarteritis score
P<0.0001
Istotypes IP / HDM IN
C
BALF cells (x 10^4)
Mucus score (cells)
Mucus score (lumen)
TSLP/IL-33 DKO + αIL-25 IP / HDM IN
D
BALF eosinophils (x 10^4)
BALF monocytes/macrophages (x 10^4)
BALF lymphoctyes (x 10^4)
BALF neutrophils (x 10^4)
E
P=0.0379
Lung tissue eosinophils (%)
F
P=0.0009
Lung tissue CD4+IL-4+ (%)
P<0.0001
Lung tissue CD4+IL-5+ (%)
P=0.0004
Lung tissue CD4+IL-13+ (%)
Lung tissue CD4+IFN-γ+ (%)
G
P=0.0369
BALF CD4+IL-5+ (%)
P=0.0335
BALF CD4+IL-13+ (%)
Isotypes IP / Saline IN
Isotypes IP / HDM IN
TSLP/IL-33 DKO + αIL-25 IP / HDM IN


Gene expression in naive liver tissue compared to Rplp2
10^1
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
Il33
Tslp
Il25


A
Gene expression in naive lung tissue compared to Rplp2
Il33
Tslp
Il25
B
P=0.002
P=0.0111
P=0.02
P=0.0144
P=0.0095
Il33 (fold)
Naive
4h
1d
8d
22d
45d
Time after initial HDM administration
P=0.0001
Tslp (fold)
Time after initial HDM administration
P=0.002
P=0.0111
P=0.0144
Il25 (fold)
Time after initial HDM administration


A
αST2, αTSLP, αIL-25
House dust mite
Harvest
Day 0 7 14 21 28 35 42 46
Hydroxyproline/lung (µmol)
P=0.0104
B
Inflammation path score
P=0.0035
Isotypes IP
αST2, αTSLP, αIL-25 IP
Endarteritis score
P=0.0339
Mucus path score
C
BALF eosinophils (x 10⁴)
BALF CD4⁺IL-4⁺ (%)
P=0.0002
BALF CD4⁺IL-5⁺ (%)
P<0.0001
nd
BALF CD4⁺IL-13⁺ (%)
P<0.0001
BALF CD4⁺IL-IFN-γ⁺ (%)
Isotypes IP / saline IN
Isotypes IP / HDM IN
αST2, αTSLP, αIL-25 / HDM IN
D
Lung tissue eosinophils (%)
P=0.0078
Tissue CD4⁺IL-4⁺ (%)
Tissue CD4⁺IL-5⁺ (%)
P=0.0076
Tissue CD4⁺IL-13⁺ (%)
P=0.0052
Tissue CD4⁺IFN-γ⁺ (%)